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Several lines of evidence suggest that accumulation of cytoplasmic β-catenin transduces an oncogenic signal. We show that β-catenin is ubiquitinated and degraded by the proteosome and that β-catenin stability is regulated by a diacylglycerol-independent protein kinase C-like kinase activity which is required for β-catenin ubiquitination. We also define a six amino acid sequence found in both β-catenin and the NF-kB regulatory protein IκBα which, upon phosphorylation, targets both proteins for ubiquitination. Mutation of a single serine within the ubiquitin targeting sequence (UTS) prevents ubiquitination of β-catenin. Mutations within the UTS of β-catenin may be oncogenic.

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FOREWORD

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Introduction

β-catenin plays an important role in both cell-cell adhesion and growth factor signal transduction. Consistent with these two functions within the cell, the protein is localized primarily in two intracellular pools; a membrane pool involved in cadherin-mediated cell-cell adhesion and a cytoplasmic pool important for signaling. The role of β-catenin in cell-cell adhesion has been well studied. It links cadherins to α-catenin and the actin cytoskeleton which results in the formation of the adherens. Tyrosine phosphorylation of β-catenin can regulate cell-cell adhesion by disrupting particular protein-protein interactions.

The *Drosophila* and *Xenopus* homologs of β-catenin are also known to be involved in signaling pathways that regulate embryonic patterning. The *Drosophila* homolog Armadillo (Arm) lies downstream of the Wingless (Wg) receptor and the serine kinase Zeste-White 3 (ZW3) in the Wingless pathway which regulates segmental pattern formation. The *Xenopus* pathway is comprised of the vertebrate homologs of the *Drosophila* proteins Wg and Zw3 (Wnt-1 and Glycogen Synthase Kinase 3β, respectively) and regulates dorsal axis formation. More recently, the interaction of β-catenin with members of the LEF/TCF family of transcription factors was shown to be important in Wnt signaling and in colon cancer.

β-catenin signaling in embryogenesis and oncogenesis appears to be regulated by controlling the accumulation of cytoplasmic β-catenin. Activation of the Wnt (Wg) pathway results in the inhibition of GSK3β (ZW3) activity which, in turn, results in stabilization of cytoplasmic β-catenin (Arm). The tumor suppressor protein APC appears to be required for the normal degradation of β-catenin as mutated forms of APC result in high levels of free (i.e. cytoplasmic) β-catenin with a longer half-life. The mechanism by which GSK3β and APC regulate β-catenin stability is unknown. Although GSK3β can directly phosphorylate APC and β-catenin *in vitro* it is not clear how these interactions regulate β-catenin stability *in vivo*.

In this report we show that β-catenin is normally degraded by the ubiquitin/proteosome pathway. We also show that certain PKC inhibitors cause a dramatic accumulation of cytoplasmic β-catenin because they inhibit its ubiquitination. In addition, we define a six amino acid motif whic is involved in targeting both β-catenin and the inibitor of NF-κB, called IκBα, for ubiquitination. A serine to alanine mutation within this ubiquitination targeting sequence (UTS) stabilizes the protein by inhibiting its ubiquitination.

Experimental Procedures

Reagents, Antibodies and Cells- ALLN (calpain inhibitor I), ALLM (calpain inhibitor II), GF-109203X (Bisindoylmaleimide), and TPA were purchased from Boehringer Mannheim. Lactacystin and Calphostin C were purchased from Calbiochem. Ro31-8220 was a gift from Dr. Robert Glazer. The monoclonal anti-β-catenin was purchased from Transduction Laboratories. The anti-ubiquitin polyclonal was raised by Dr. Weissman. The anti-HA was monoclonal clone HA-11 was from BabCo. SKBR3 and HBL100 cell lines were acquired from the ATCC and maintained in DMEM with 10% FBS and 1% penicillin/streptomycin.

Cellular Fractionation- NP-40 extractions were performed as described previously {1401} except that 10 mM N-ethylmaleimide (NEM) was added to the lysis buffer. Cytoplasmic fractionation was performed by washing cells twice in PBS and removing all remnants of the final wash. The cells were incubated in ice cold hypotonic lysis buffer (10 mM Tris, pH 7.4, 0.2 mM $MgCl_2$, 5 mM NEM and 10 mM ALLN) for 5 minutes on ice. The cells were scraped and mechanically disrupted by 30 strokes in a Dounce homogenizer. Greater than 95% of the cells were lysed as judged by light microscopy. The lysates were transferred to an ultracentrifuge tube containing a 5X inhibitor solution (250 mM NaF, 5 mM Na vanadate, 25 mM NEM, aprotinin, leupeptin, pepstatin A, and AEBSF). The insoluble components of the lysates were pelleted at 100,000 x g for 1 hour. The supernatant was designated the S100 or cytoplasmic fraction and the pellet was the P100 fraction. In some experiments the P100 fraction was extracted with NP-40 buffer. Lysates were boiled in 2X Laemlli sample buffer with 10% β-mercaptoethanol and separated on 8% or 4-12% polyacrylamide gels (Novex).

Immunoprecipitations- Immunoprecipitations were performed on equal amounts of NP-40 lysates. The lysates were pre-cleared once with 10 mg non-immune mouse IgG and 100 ml protein G/Sepharose (Gibco) and once with 100 ml of protein G/Sepharose, both for 1.5 hours in the cold. The lysates were incubated in the cold for 1.5 hours with 6 mg non-immune mouse IgG or monoclonal anti-β-catenin. Protein G/Sepharose was added for 45 minutes. The Protein G/Antibody/antigen complexes were pelleted in a cold microcentrifuge and washed six times in cold NP-40 buffer without inhibitors. The immunoprecipitated proteins were boiled for five minutes and separated by SDS-PAGE. The proteins were transferred to PVDF (Millipore).

β-catenin construct and mutagenesis- The nine amino acid HA-tag was added to the C-terminus of β-catenin using PCR. The β-catenin/HA construct was cloned into the BamHI site of the pcDNA3 vector (Invitrogen). Mutagenesis of serine 37 was performed using the Quick-Change Site-directed Mutagenesis Kit (Stratagene).

Transfections- Transfections were performed by $CaPO_4$ precipitation. $CaPO_4$/DNA precipitate was incubated with the cells for 6 hours. SKBR3 cells were shocked with 20% glycerol in serum-free medium for 4 minutes before washing and then the medium was replaced. Assays were done 24 to 72 hours after transfection.

Results

β-catenin is degraded by the ubiquitini-proteosome pathway. In order to more fully define the mechanisms by which β-catenin signaling is regulated, we set out to determine how the protein is normally degraded. Proteins which are to be degraded by the ubiquitin/proteosome system are first conjugated to multiple copies of the small protein ubiquitin through isopeptide linkages. These ubiquitinated proteins are then recognized and degraded by the 26S proteosome. Inhibition of the proteosome results in the accumulation of the ubiquitinated forms of those proteins normally degraded by the proteosome. In order to determine whether β-catenin is degraded by this system, human breast epithelial cell lines were treated with the peptide aldehyde proteosomal inhibitor ALLN or a related peptide aldehyde that has 50 to 100-fold lower potency as a proteosomal inhibitor. Anti-β-catenin immunoblots revealed that the proteosomal

inhibitor at a dose as low as 1 μM resulted in the accumulation of β-catenin including the appearance of high molecular weight (HMW) β-catenin species. The same result was found using the specific proteosomal inhibitors lactacystin. These experiments were conducted with both SKBR3 and HBL100 cell lines with similar results.

In order to show that β-catenin is ubiquitinated before proteosomal degradation, we immunoprecipitated β-catenin from HBL100 cells treated with either ALLN or vehicle (DMSO) alone and detected ubiquitinated material by immunoblot. Only when cells were treated with the proteosomal inhibitor did a significant amount of ubiquitin immunoreactivity co-precipitate with β-catenin indicating that it is conjugated to ubiquitin prior to degradation by the proteosome. These data demonstrate unequivocally that β-catenin is normally degraded by the proteosome following ubiquitination.

An atypical PKC-like kinase regulates cytoplasmic β-catenin accumulation. One serine kinase is clearly an important regulator of β-catenin accumulation. GSK3β is a member of the Wnt/Wg pathway and its activity is required to maintain low levels of cytoplasmic β-catenin. In addition, GSK3β itself can be regulated by several other serine kinases. *In vitro,* p70 S6 kinase, p90[Rsk], Akt/protein kinase B (PKB) and certain protein kinase C (PKC) isoforms phosphorylate and regulate GSK3β activity. Several other growth factors including EGF and IGF also result in the inactivation of GSK3β. In cultured cells, inhibitor studies showed that the activity of a TPA-sensitive PKC isoform is required to inactivate GSK3β in response to the Wg signal. This PKC isoform appeared to be specifically involved in Wg-mediated regulation of GSK3β as it was not involved in other GSK3β regulating signals (e.g. insulin, EGF, IGF-1, and serum). No studies have determined the effect of PKC inhibition on β-catenin accumulation.

In order to determine the role of PKC in the regulation of β-catenin stability, cells were treated with different PKC inhibitors and assayed for β-catenin accumulation. In the HBL100 cell line which has high levels membrane-associated and low levels of cytoplasmic β-catenin, the two bisindoylmaleimide-type PKC inhibitors (GF-109203X and Ro31-8220) caused a dramatic increase in the cytoplasmic pool but not the membrane pool. The same result was seen in MDCK cells. In SKBR3 cells which have very low levels of β-catenin protein in both pools, there was a large increase in both cytoplasmic and membrane (NP-40 soluble) pools. PKC activity is required for Wnt-1 signaling to inhibit GSK3β activity. Although this study did not investigate the effect of PKC inhibitors on β-catenin stability, one would anticipate that inhibition of PKC would have resulted in decreased β-catenin stability. TPA-induced down regulation of DAG-dependent PKCs also prevented Wnt from inhibiting GSK3β. In contrast, neither the PKC inhibitor Calphostin C nor TPA-induced down regulation of PKCs had a significant effect on the accumulation of cytoplasmic β-catenin in the present study. In this experiment, TPA treatment completely down regulated PKCα, the major PKC isoform expressed in these cells. Our interpretation of these results is based on the different mechanisms of the inhibitors. The bisindoylmaleimides inhibit both DAG-dependent and -independent PKC isoforms by competing with ATP for binding to the kinase whereas calphostin C and long-term phorbol ester treatment inhibit only DAG-dependent PKC activities. It should be noted that the bisindoylmaleimide Ro31-8220 has no direct effect on GSK3β activity and actually acts to prevent the decrease in activity normally induced

by Wg. The inhibitor profile of the PKC isoform involved in β-catenin accumulation indicates that it may be a member of the DAG-independent class of PKCs known as atypical PKCs (aPKC). These results distinguish it from the DAG-dependent PKC isoform that is responsible for Wnt-dependent GSK3β regulation.

In addition to inhibitors of PKC, lithium chloride, which is an inhibitor of GSK3β (as well as other enzymes), was used to investigate its effects on β-catenin accumulation. Treatment of *Xenopus* embryos with lithium chloride results in the formation of a secondary dorsal axis in the embryo, phenotypes which are also characteristic of ectopic Wnt or β-catenin overexpression. Treatment with 30 m$\underline{M}$ LiCl results in the accumulation of cytoplasmic β-catenin within HBL100 and SKBR3 cells. This suggests that the effects of lithium that are seen in *Xenopus* embryos are a result of increased cytoplasmic β-catenin.

GF-109203X and lithium inhibit ubiquitination of β-catenin. The bisindoylmaleimide PKC inhibitors and lithium may increase cytoplasmic β-catenin by inhibiting its ubiquitination and, therefore, its degradation. To investigate this possibility, SKBR3 and HBL100 cells were treated overnight with the proteosomal inhibitor ALLN in order to generate the HMW ubiquitin-conjugated forms of β-catenin. In addition to the proteosomal inhibitor, we treated some cells with inhibitory or non-inhibitory doses of the PKC inhibitor GF-109203X and LiCl. The reduction or complete abrogation of β-catenin ubiquitination by these inhibitors demonstrates that the aPKC-like activity and GSK3β, respectively, are required for ubiquitination of β-catenin. This parallels other work regarding the role of GSK3β in regulating β-catenin degradation and introduces the possibility that an atypical PKC-like enzyme may also play a role in this important process.

A conserved six-amino acid sequence targets β-catenin and IκBα for ubiquitination. Since N-terminal truncated forms of β-catenin and Arm accumulate in the cytoplasm, we analyzed the N-terminus of β-catenin for serine residues that might be involved in regulating β-catenin ubiquitination. Five N-terminal serines that are well conserved between β-catenin, *Armadillo,* and plakoglobin are present between β-catenin amino acids 29 and 47. Mutation of three of these serines as well as a conserved threonine results in β-catenin accumulation and axis duplication in *Xenopus*. Interestingly, two of these serines lie within a six amino acid region that is almost identical to a motif in the protein IκBα which, upon serine phosphorylation, targets it for ubiquitination. Mutations of one or both of the serines in this motif stabilize IκBα by inhibiting its ubiquitination. Mutations of β-catenin serine 37 were recently reported to occur in several melanoma cell lines. In order to determine the role that phosphorylation of this motif within β-catenin might play in its ubiquitination and degradation, a serine to alanine mutation was made at residue 37 of β-catenin.

The wild-type and mutant β-catenin constructs were transfected into SKBR3 cells. The cytoplasmic proteins (S100) were isolated from the other cellular proteins (P100) and both fractions separated by SDS-PAGE and immunoblotted for β-catenin. The S37A mutant β-catenin accumulated to approximately three-fold the levels of the wild-type 36 hours after transfection. Importantly, this differential accumulation was

only detected in the cytoplasmic pool of β-catenin as the P100 fraction shows approximately equal accumulation of the two forms of β-catenin.

In order to determine whether this increased accumulation was due to reduced ubiquitination of the mutant, SKBR3 cells were transfected with wild-type, the S37A mutant, or vector alone. The transfectants were lysed and assayed for the accumulation of HMW ubiquitinated β-catenin by immunoblotting with an anti-HA antibody. The wild-type β-catenin was ubiquitinated efficiently while the mutant form was not ubiquitinated. Interestingly, ubiquitinated β-catenin only accumulated in the S100 fraction of the SKBR3 cell line. This result strongly suggests that, like IκBα, a specific serine (residue 37, in β-catenin) must be phosphorylated prior to ubiquitination of β-catenin. Phosphorylation of this serine is probably an important mechanism by which the amount of β-catenin within the cell is regulated. Indeed, mutations of this serine and serine 33, which is also located within the UTS, occur in several melanoma and colon cancer cell lines. In these cases, the mutant forms of β-catenin were presumed to be transforming.

These data show that β-catenin is normally degraded by the ubiquitin/proteosome system and that at least two serine/threonine kinases regulate this process, GSK3β and an aPKC-like enzyme. We also identify a common motif within β-catenin and IκBα which, upon being phosphorylated, regulates the stability of these proteins by targeting them for ubiquitination.

The identity of the kinase(s) which phosphorylates the serines in the IκBα UTS is unknown. The aPKC-like kinase activity which regulates β-catenin ubiquitination may directly phosphorylate the UTS or it may be involved in the regulation of the kinase that does. Regardless of the specific interactions, it is likely that signaling through β-catenin is regulated by a complex network of kinases which regulate its ubiquitination.

The regulation of β-catenin signaling *per se* may well be an important mechanism by which multiple intracellular signals are integrated. We propose that several signaling pathways, such as signaling through growth factors (e.g. the Wnts, EGF, IGF, insulin), APC, and retinoic acid-induced adhesion, may converge by regulating the accumulation of cytoplasmic β-catenin. Several conserved serines and threonines are present within a small region of the N-terminus of β-catenin. Since four of these residues are targets for mutation in colon cancers and melanomas, it is likely that many, if not all, of these residues must be phosphorylated in order to target β-catenin for degradation. One possible scenario is that these kinases function in a specific temporal sequence. In this model, kinase 2 might require the phosphorylation of a certain residue by kinase 1 in order for it to recognize its target residues. Alternatively, these kinases may function independently of each other. In either instance we propose that as the cellular context changes β-catenin phosphorylation will be altered and these alterations will determine whether or not it is ubiquitinated and degraded.

The definition of a specific region that targets an oncogenic protein for degradation may prove to be useful in the design of modalities for cancer therapy. We hypothesize that the UTS will be recognized by β-catenin-directed ubiquitin-conjugating enzymes which may themselves constitute therapeutic targets.

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List of Publications

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Communication

Serine Phosphorylation-regulated Ubiquitination and Degradation of β-Catenin*

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Several lines of evidence suggest that accumulation of cytoplasmic β-catenin transduces an oncogenic signal. We show that β-catenin is ubiquitinated and degraded by the proteosome and that β-catenin stability is regulated by a diacylglycerol-independent protein kinase C-like kinase activity, which is required for β-catenin ubiquitination. We also define a six-amino acid sequence found in both β-catenin and the NF-κB regulatory protein IκBα, which, upon phosphorylation, targets both proteins for ubiquitination. Mutation of a single serine within the ubiquitination targeting sequence prevents ubiquitination of β-catenin. Mutations within the ubiquitination targeting sequence of β-catenin may be oncogenic.

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[1] The abbreviations used are: Arm, Armadillo; Wg, Wingless; ZW3, Zeste-White 3; GSK3β, glycogen synthase kinase-3β; PKC, protein kinase C; UTS, ubiquitination targeting sequence; ALLN, N-acetyl-Leu-Leu-norleucinal; ALLM, N-Acetyl-Leu-Leu-methional; TPA, 12-O-tetradecanoylphorbol 13-acetate; NEM, N-ethylmaleimide; HMW, high molecular weight; aPKC, atypical protein kinase C; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; EGF, epidermal growth factor; IGF, insulin-like growth factor; DAG, diacylglycerol.
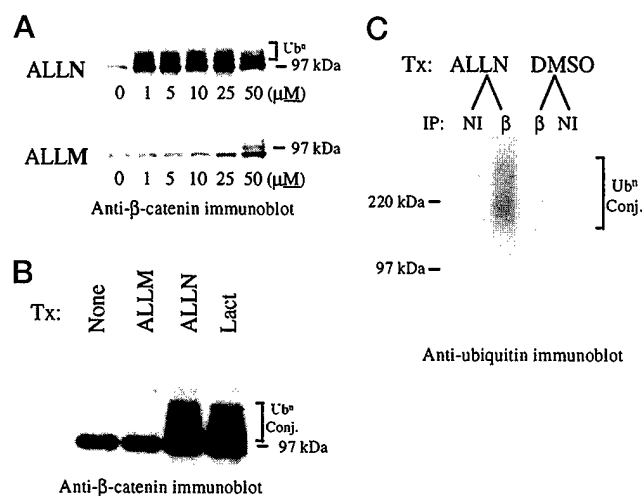
FIG. 1. **β-Catenin is ubiquitinated and degraded by the proteosome.** Immunoblots and immunoprecipitations were performed with equal amounts of Nonidet P-40 lysates. *A*, SKBR3 cells were treated for 4 h with increasing amounts of either the peptide aldehyde proteosomal inhibitor ALLN or the related peptide aldehyde calpain protease inhibitor ALLM. Cells were lysed in Nonidet P-40, separated by SDS-PAGE, and immunoblotted with an anti-β-catenin monoclonal antibody. *B*, SKBR3 cells were treated *(Tx)* overnight with the proteosomal inhibitors ALLN (10 μM) and lactacystin *(Lact.)* (100 μM) and with ALLM (10 μM) and the lactacystin vehicle ethanol (20 μl). The cells were lysed and analyzed as in *A*. *C*, HBL100 cells were treated overnight with either 10 μM ALLN or vehicle (Me₂SO *(DMSO)*) overnight. The cells were lysed in Nonidet P-40 and immunoprecipitated *(IP)* with 6 μg of either anti-β-catenin monoclonal antibody *(β)* or non-immune mouse IgG *(NI)*. The precipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride. The membrane was treated as in Ref. 15 and probed with an anti-ubiquitin antibody.
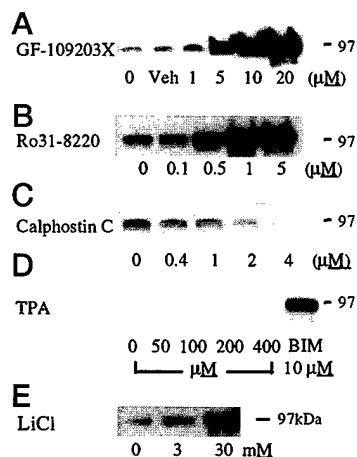
FIG. 2. **Cytoplasmic β-catenin accumulates in response to bisindoylmaleimide PKC inhibitors and lithium.** Confluent plates of HBL100 s *(A, B, C,* and *E)* were treated overnight with the PKC inhibitors GF-109203X *(A)*, Ro31-8220 *(B)*, and calphostin C *(C)*, and the pluripotent inhibitor lithium chloride *(E)* at increasing doses. SKBR3 *(D)* cells were treated with increasing doses of PMA for 3 days. Cytoplasmic extracts were made from all of these cells and the proteins separated by SDS-PAGE and immunoblotted with anti-β-catenin monoclonal antibody. Equal amounts of cytoplasmic extracts were loaded per well for immunoblotting. Calphostin C was toxic to the cells at 4 μM.

The immunoprecipitated proteins were boiled for 5 min and separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (Millipore) and treated as described in Ref. 15.

β-Catenin Construct and Mutagenesis — The nine-amino acid HA-tag was added to the C terminus of β-catenin using polymerase chain reaction. The β-catenin/HA construct was cloned into the *Bam*HI site of the pcDNA3 vector (Invitrogen). Mutagenesis of serine 37 was performed using the Quick-Change site-directed mutagenesis kit (Stratagene).
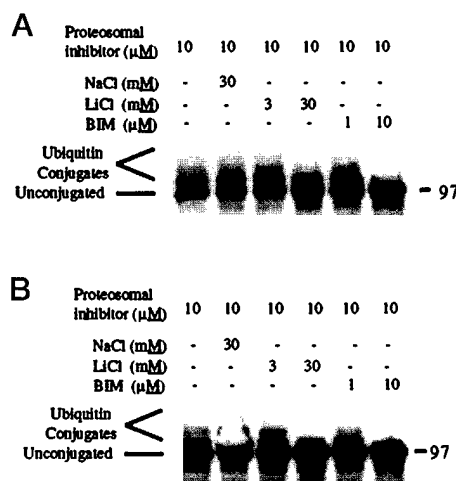
FIG. 3. **GF-109203X and lithium inhibit β-catenin ubiquitination.** Immunoblots were performed with equal amounts of Nonidet P-40 lysates. Confluent plates of SKBR3s *(A)* and HBL100s *(B)* were treated with ALLN (10 μM) overnight. In addition, cells were treated with: control *(lane 1)*, 30 mM NaCl, 3 mM LiCl, 30 mM LiCl, 1 μM GF-109203X, or 10 μM GF-109203X. The cells were lysed and fractionated as in Fig. 1*A*. Immunoblotting was performed with monoclonal anti-β-catenin.
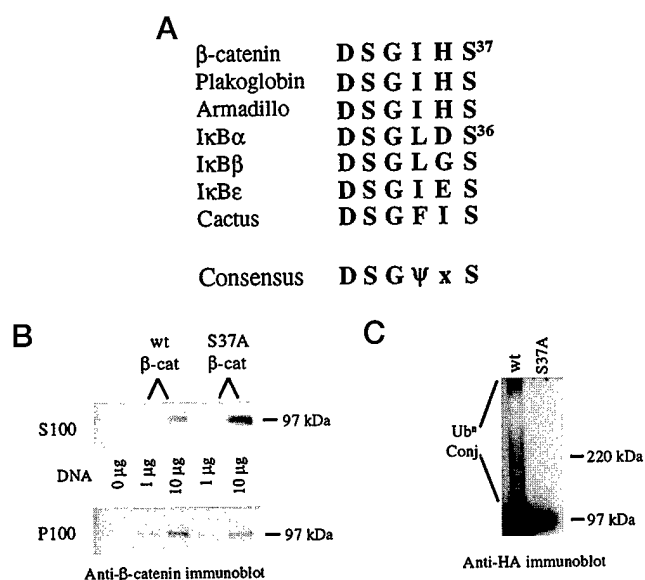
Transfections — Transfections were performed by calcium-phosphate precipitation (16). The calcium-phosphate/DNA precipitate was incubated with the cells for 6 h. SKBR3 cells were shocked with 20% glycerol in serum-free medium for 4 min before washing and then the medium was replaced. Assays were performed 24–72 h after transfection.

RESULTS AND DISCUSSION

β-Catenin Is Degraded by the Ubiquitin-Proteosome Pathway — Proteins that are to be degraded by the ubiquitin/proteosome system are first conjugated to multiple copies of the small protein ubiquitin through isopeptide linkages (17). These ubiquitinated proteins are then recognized and degraded by the 26 S proteosome. Inhibition of the proteosome results in the accumulation of the ubiquitinated forms of those proteins normally degraded by the proteosome. To determine whether β-catenin is degraded by this system, human breast epithelial cell lines were treated with the peptide aldehyde proteosomal inhibitor ALLN or a related peptide aldehyde that has 50–100-fold lower potency as a proteosomal inhibitor. Anti-β-catenin immunoblots revealed that the proteosomal inhibitor at a dose as low as 1 μM resulted in the accumulation of β-catenin, including the appearance of high molecular weight (HMW) β-catenin species (Fig. 1*A*). The same result was found using the specific proteosomal inhibitor lactacystin (Fig. 1*B*). These experiments were conducted with both SKBR3 and HBL100 cell lines with similar results.

To show that β-catenin is ubiquitinated before proteosomal degradation, we immunoprecipitated β-catenin from HBL100 cells treated with either ALLN or vehicle (Me₂SO) alone and detected ubiquitinated material by immunoblot. Only when cells were treated with the proteosomal inhibitor did a significant amount of ubiquitin immunoreactivity co-precipitate with β-catenin, indicating that it is conjugated to ubiquitin prior to degradation by the proteosome. These data demonstrate unequivocally that β-catenin is normally degraded by the proteosome following ubiquitination.

An Atypical PKC-like Kinase Regulates Cytoplasmic β-Catenin Accumulation — GSK3β is a member of the Wnt/Wg pathway, and its activity is required to maintain low levels of cytoplasmic β-catenin (9, 12). In addition, GSK3β itself can be regulated by several other serine kinases. *In vitro,* p70 S6 kinase, p90[Rsk], Akt/protein kinase B (PKB), and certain PKC isoforms phosphorylate and regulate GSK3β activity (18–21).

A

β-catenin	D S G I H S^{37}
Plakoglobin	D S G I H S
Armadillo	D S G I H S
IκBα	D S G L D S^{36}
IκBβ	D S G L G S
IκBε	D S G I E S
Cactus	D S G F I S

Consensus D S G Ψ x S

B

C

Anti-β-catenin immunoblot

Anti-HA immunoblot

FIG. 4. **β-Catenin contains an IκBα-like six-amino acid sequence, which, when phosphorylated, targets β-catenin for ubiquitination.** *A*, the six-amino acid motif is well conserved in both IκBα and β-catenin family members. *B*, either 0, 1, or 10 μg of wild-type and S37A mutant β-catenin constructs were transfected into SKBR3 cells. 36 h later, the cells were fractionated into S100 (cytoplasmic) and P100 (pellet) pools. The proteins were separated by SDS-PAGE and immunoblotted with monoclonal anti-β-catenin antibody as in Fig. 2. *C*, 10 μg of wild-type β-catenin or S37A mutant β-catenin were transfected into SKBR3 cells. 30 h later, cells were treated with ALLN (5 μM) and clasto-lactacystin (5 μM) overnight. The cells were fractionated into S100 and P100 pools, and the cytoplasmic proteins were separated by SDS-PAGE and immunoblotted with anti-HA antibody. Note the accumulation of HMW HA-tagged wild-type, but not mutant, β-catenin in the cytoplasmic fraction. Cells transfected with vector alone showed no anti-HA immunoreactivity (data not shown).

A

B

^{27}QQSYLDSGIHSGATTTAPSLSGKG^{50}

FIG. 5. **A complex array of kinases regulates β-catenin stability.** *A*, inhibitor studies from Cook *et al.* (23) and the present work indicate that several serine kinases are likely to be important in the regulation of β-catenin ubiquitination. A DAG-dependent classical PKC is required to mediate the inhibitory effects of Wnt/Wg on GSK3β activity. It should be noted that other serine kinases can also phosphorylate and inactivate GSK3β. Active GSK3β can phosphorylate APC and β-catenin. The present study implicates an additional DAG-independent PKC-like activity in the modulation of β-catenin ubiquitination and stability. *B*, conserved serine and threonine residues in the ubiquitin-targeting sequence at the N-terminal of β-catenin may also be the target of several kinases. These could act sequentially or in concert to prepare β-catenin for ubiquitination and/or interaction with the ubiquitin-conjugating machinery. Mutation of any one of these serines may be transforming. It is not clear if all of these serines need to be phosphorylated in order for β-catenin to be ubiquitinated, but it is clear that mutation of a single residue (S37A) is sufficient to prevent ubiquitination.

Several other growth factors, including EGF and IGF also result in the inactivation of GSK3β (22). In cultured cells, inhibitor studies showed that the activity of a TPA-sensitive PKC isoform is required to inactivate GSK3β in response to the Wg signal (23). This PKC isoform appeared to be specifically involved in Wg-mediated regulation of GSK3β, as it was not involved in other GSK3β regulating signals (*e.g.* insulin, EGF, IGF-1, and serum) (23). No studies have determined the effect of PKC inhibition on β-catenin accumulation.

Cells were treated with different PKC inhibitors and assayed for β-catenin accumulation. In the HBL100 cell line, which has high levels of membrane-associated and low levels of cytoplasmic β-catenin, two bisindoylmaleimide-type PKC inhibitors (GF-109203X and Ro31-8220) caused a dramatic increase in the cytoplasmic pool but not the membrane pool (Fig. 2*B*). The same result was seen in Madin-Darby canine kidney cells (data not shown). In SKBR3 cells, which have very low levels of β-catenin protein in both pools, there was a large increase in both cytoplasmic and membrane (Nonidet P-40-soluble) pools (data not shown). PKC activity is required for Wnt-1 signaling to inhibit GSK3β activity (23). Although this study did not investigate the effect of PKC inhibitors on β-catenin stability, one would anticipate that inhibition of PKC would have resulted in decreased β-catenin stability. TPA-induced down-regulation of DAG-dependent PKCs also prevented Wnt from inhibiting GSK3β (23). In contrast, neither the PKC inhibitor calphostin C nor TPA-induced down-regulation of PKCs had a significant effect on the accumulation of cytoplasmic β-catenin in the present study (Fig. 2, *C* and *D*). In this experiment, TPA treatment completely down-regulated PKCα, the major PKC isoform expressed in these cells (data not shown). Our inter-

pretation of these results is based on the different mechanisms of the inhibitors. The bisindoylmaleimides inhibit both DAG-dependent and -independent PKC isoforms by competing with ATP for binding to the kinase, whereas calphostin C and long term phorbol ester treatment inhibit only DAG-dependent PKC activities. It should be noted that the bisindoylmaleimide Ro31-8220 has no direct effect on GSK3β activity and actually acts to prevent the decrease in activity normally induced by Wg (23). The inhibitor profile of the PKC isoform involved in β-catenin accumulation indicates that it may be a member of the DAG-independent class of PKCs known as atypical PKCs (aPKC). These results distinguish it from the DAG-dependent PKC isoform that is responsible for Wnt-dependent GSK3β regulation (23).

In addition to inhibitors of PKC, lithium chloride, which is an inhibitor of GSK3β (as well as other enzymes), was used to investigate its effects on β-catenin accumulation (24, 25). Treatment of *Xenopus* embryos with lithium chloride results in the formation of a secondary dorsal axis in the embryo, phenotypes that are also characteristic of ectopic Wnt or β-catenin overexpression (2, 26). Treatment with 30 mM results in the accumulation of cytoplasmic β-catenin within HBL100 and SKBR3 cells (Fig. 2*E*). This suggests that the effects of lithium that are seen in *Xenopus* embryos are a result of increased cytoplasmic β-catenin.

GF-109203X and Lithium Inhibit Ubiquitination of β-Catenin—The bisindoylmaleimide PKC inhibitors and lithium may increase cytoplasmic β-catenin by inhibiting its ubiquitination and, therefore, its degradation. To investigate this possibility, SKBR3 and HBL100 cells were treated overnight with the

proteosomal inhibitor ALLN to generate the HMW ubiquitin-conjugated forms of β-catenin. In addition to the proteosomal inhibitor, we treated some cells with inhibitory or non-inhibitory doses of the PKC inhibitor GF-109203X and LiCl. The reduction or complete abrogation of β-catenin ubiquitination by these inhibitors demonstrates that the aPKC-like activity and GSK3β, respectively, are required for ubiquitination of β-catenin (Fig. 3). This parallels other work regarding the role of GSK3β in regulating β-catenin degradation and introduces the possibility that an atypical PKC-like enzyme may also play a role in this important process.

A Conserved Six-amino Acid Sequence Targets β-Catenin and IκBα for Ubiquitination — Since N-terminal truncated forms of β-catenin and Arm accumulate in the cytoplasm, we analyzed the N terminus of β-catenin for serine residues that might be involved in regulating β-catenin ubiquitination (27). Five N-terminal serines that are well conserved between β-catenin, *Armadillo*, and plakoglobin are present between β-catenin amino acids 29 and 47. Mutation of three of these serines as well as a conserved threonine results in β-catenin accumulation and axis duplication in *Xenopus* (12). Interestingly, two of these serines lie within a six-amino acid region that is almost identical to a motif in the protein IκBα, which, upon serine phosphorylation, targets it for ubiquitination (see Fig. 4A) (28). Mutations of one or both of the serines in this motif stabilize IκBα by inhibiting its ubiquitination (28). Mutations of β-catenin serine 37 were recently reported to occur in several melanoma cell lines (29). To determine the role that phosphorylation of this motif within β-catenin might play in its ubiquitination and degradation, a serine to alanine mutation was made at residue 37 of β-catenin.

The wild-type and mutant β-catenin constructs were transfected into SKBR3 cells. The cytoplasmic proteins (S100) were isolated from the other cellular proteins (P100), and both fractions separated by SDS-PAGE and immunoblotted for β-catenin. The S37A mutant β-catenin accumulated to approximately 3-fold the levels of the wild-type 36 h after transfection (Fig. 4B). This differential accumulation was only detected in the cytoplasmic pool of β-catenin as the P100 fraction shows approximately equal accumulation of the two forms of β-catenin.

To determine whether this increased accumulation was due to reduced ubiquitination of the mutant, SKBR3 cells were transfected with wild-type, the S37A mutant, or vector alone. The transfectants were lysed and assayed for the accumulation of HMW ubiquitinated β-catenin by immunoblotting with an anti-HA antibody (Fig. 4C). The wild-type β-catenin was ubiquitinated efficiently, while the mutant form was not ubiquitinated. Interestingly, ubiquitinated β-catenin only accumulated in the S100 fraction of the SKBR3 cell line (data not shown). This result strongly suggests that, like IκBα, a specific serine (residue 37, in β-catenin) must be phosphorylated prior to ubiquitination of β-catenin. Phosphorylation of this serine is probably an important mechanism by which the amount of β-catenin within the cell is regulated. Indeed, mutations of this serine and serine 33, which is also located within the UTS, occur in several melanoma and colon cancer cell lines (8, 29). In these cases, the mutant forms of β-catenin were presumed to be transforming.

These data show that β-catenin is normally degraded by the ubiquitin/proteosome system and that at least two serine/threonine kinases regulate this process, GSK3β and an aPKC-like enzyme. We also identify a common motif within β-catenin and IκBα, which, upon being phosphorylated, regulates the stability of these proteins by targeting them for ubiquitination. The identity of the kinase(s) that phosphorylates the IκBα UTS is unknown. The aPKC-like kinase activity that regulates β-cate-

nin ubiquitination may directly phosphorylate the UTS, or it may be involved in the regulation of the kinase that does. Regardless of the specific interactions, it is likely that signaling through β-catenin is regulated by a complex network of kinases that regulate its ubiquitination (Fig. 5A).

The regulation of β-catenin signaling *per se* may well be an important mechanism by which multiple intracellular signals are integrated. We propose that several signaling pathways, such as signaling through growth factors (*e.g.* the Wnts, EGF, IGF, insulin), APC, and retinoic acid-induced adhesion, may converge by regulating the accumulation of cytoplasmic β-catenin (Fig. 5B) (10, 14). Several conserved serines and threonines within a small region of the N terminus of β-catenin are targets for mutation in colon cancers and melanomas. It is likely that many, if not all, of these residues must be phosphorylated to target β-catenin for degradation (8, 29). We propose that as the cellular context changes, β-catenin phosphorylation will be altered, and these alterations will determine whether or not it is ubiquitinated and degraded.

The definition of a specific region that targets an oncogenic protein for degradation may prove to be useful in the design of modalities for cancer therapy. We hypothesize that the UTS will be recognized by β-catenin-directed ubiquitin-conjugating enzymes, which may themselves constitute therapeutic targets.

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